Investigations into the temporal development of epitheliocystis infections in brown trout: a histological study

Guevara Soto, M ; Vidondo, B ; Vaughan, L ; Rubin, J-F ; Segner, H ; Samartin, S ; Schmidt-Posthaus, H

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Temporal development of epitheliocystis infections in brown trout (Salmo trutta) during the course of the year and comparison between consecutive years

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Complete List of Authors:	Guevara, Maricruz; University of Bern, Centre for Fish and Wildlife Health Vidondo, Beatriz; Institute of Veterinary Public Health, University of Bern Vaughan, Lloyd; Institute for Veterinary Pathology, Vetsuisse Faculty, University of Zurich Rubin, Jean-Francois; Fondation de la Maison de la Rivière Segner, Helmut; Universitat Bern Zentrum fur Fisch- und Wildtiermedizin Samartin, Stéphanie; Institute of Veterinary Public Health Schmidt-Posthaus, Heike; University of Bern, Centre for Fish and Wildlife Health

Keywords:	epitheliocystis, brown trout, temperature, season, inter-year comparison

Temporal development of epitheliocystis infections in brown trout (*Salmo trutta*) during the course of the year and comparison between consecutive years

Maricruz Guevara Soto[1*], Beatriz Vidondo[2*], Lloyd Vaughan[3], Jean-Francois Rubin[4], Helmut Segner[1], Stéphanie Samartin[2], Heike Schmidt-Posthaus[1*]

[1]Centre of Fish and Wildlife Health, Department of Infectious Diseases and Pathobiology, University of Bern, Switzerland, [2]Institute of Veterinary Public Health, University of Bern, Switzerland, [3]Institute of Veterinary Pathology, University of Zurich, Switzerland, and [4]Fondation de la Maison de la Rivière, Tolochenaz, Switzerland

*These authors contributed equally

Corresponding author: heike.schmidt@vetsuisse.unibe.ch, Tel.: +41 31 631 24 65, fax: +41 31 631 26 11

Running title: temporal development of epitheliocystis in brown trout

Abstract

Epitheliocystis in Swiss brown trout (*Salmo trutta*) is a chlamydial infection, mainly caused by *Candidatus* Piscichlamydia salmonis and *Candidatus* Clavichlamydia salmonicola. To gain a better understanding of the temporal development of infections in wild brown trout, we investigated epitheliocystis infections during the course of the summer and autumn months of a single year (2015), and compared this to sampling points over the span of the years 2012-2014. The survey focused on tributaries (Venoge and Boiron) of the Rhone flowing in to Lake Geneva. When evaluated histologically, epitheliocystis infections were found throughout the period of investigation with the exception of the month of June. 50 to 86 animals per sampling were investigated. Highest prevalences and infection intensities were seen in September. A correlation between epitheliocystis infection and water temperatures was not evident. Inter-year comparison revealed consistent levels of prevalence and infection intensities in late summer. The absence of infections in June, combined with the consistent inter-year results, indicate seasonal fluctuation of epitheliocystis infections in brown trout with a reservoir during winter months from which infections can re-initiate each year. This could either be at levels below detection limit within the brown trout population itself or in an alternative host.

Keywords: epitheliocystis, brown trout, temperature, season, inter-year comparison

Introduction

Epitheliocystis is a bacterial disease caused by members of the phylum *Chlamydiae,* but also by γ- and β-proteobacteria (Kurahashi & Yokota 2006; Toenshoff, Kvellestad, Mitchell, Steinum, Falk, Colquhoun & Horn 2012; Mendoza, Guiza, Martinez, Caraballo, Rojas, Aranguren & Salazar 2013; Katharios, Seth-Smith, Fehr, Mateos, Qi, Richter, Nufer, Ruetten, Guevara Soto, Ziegler, Thomson, Schlapbach & Vaughan 2015; Seth-Smith, Dourala, Fehr, Katharios, Qi, Ruetten, Mateos, Nufer, Weilenmann, Ziegler, Thomson, Schlapbach &Vaughan 2016; Qi, Vaughan, Katharios, Schlapbach & Seth-Smith 2016). It affects more than 90 wild and farmed fish species worldwide, both in marine and fresh-water (Paperna & Sabnai 1980; Lewis, McLaughlin, Bodammer & Sawyer 1992; Nowak & La Patra 2006; Abowei & Briyani 2011; Stride, Polkinghorne, Miller, Groff, LaPatra & Nowak 2013 a; Stride, Polkinghorne, Miller & Nowak 2013 b; Stride, Polkinghorne & Nowak 2014). It was initially described as lesions within the gill and skin epithelium of bluegills (*Lepomis macrochirus*) (Hoffman, Dunbar, Wolf & Zwillenberg 1969), with associated hypertrophy of infected epithelial cells and formation of basophilic granule filled spherical cysts. In infections of salmonids of the genus *Salmo* and *Salvelinus,* four different chlamydial species were identified so far*, Candidatus* Piscichlamydia salmonis, *Candidatus* Clavichlamydia salmonicola and *Candidatus* Similichlamydia sp. (Draghi, Popov, Kahl, Stanton, Brown, Tsongalis, West & Frasca 2004; Draghi, Bebak, Daniel, Tulman, Geary, West, Popov & Frasca 2010; Mitchell, Steinum, Rodger, Holland, Falk & Colquhoun 2010; Schmidt-Posthaus, Polkinghorne, Nufer, Schifferli, Zimmermann, Segner, Steiner & Vaughan 2012; Mitchell, Steinum, Toenshoff, Kvellestad, Falk, Horn & Colquhoun 2013; Contador, Methner, Ryerse, Huber, Lillie, Frasca & Lumsden 2015; Guevara Soto, Vaughan, Segner, Wahli, Vidondo & Schmidt-Posthaus 2016 b) along with the β-proteobacteria *Candidatus* Brachiomonas cysticola (Toenshoff et al. 2012). Macroscopically, infections in salmonids are not visible to the naked eye, with cysts typically between 10-20μm in diameter (Schmidt-Posthaus et al. 2012; Guevera Soto et al. 2016 a, b) and only detectable histologically. Whereas *Ca.* P. salmonis and *Ca*. B. cysticola are found in both fresh water and sea water hosts, *Ca.* C. salmonicola appears to be fresh water specific, found in farmed salmon and in wild brown trout (Karlsen, Nylund, Watanabe, Helvik, Nylund & Plarre 2008; Mitchell et al. 2010), and is lost upon transfer of infected farmed salmon to marine cages (Mitchell et al. 2010). Whether the recently identified *Ca*. Simichlamydia sp. (Guevera Soto et al. 2016 b) are freshwater specific, cannot be answered yet. The closest relative to the strain found in Swiss brown trout is *Ca*. Similichlamydia labra sp. nov., a species recently identified in ballan wrasse (*Labrus* *bergylta*) used as cleaner fish on Atlantic salmon in sea water cages (Steigen, Karlsbakk, Plarre, Watanabe, Øvergård, Brevik & Nylund 2015). However, infections of salmon in the same environment were not mentioned. Until the recent report, infections with *Ca*. Similichlamydia sp. were restricted to marine hosts and no infections in salmonids have been described before (Steigen et al. 2015; Stride et al. 2013a,b).

In Switzerland, epitheliocystis has been found to be widely distributed in wild brown trout populations (Schmidt-Posthaus, Bernet, Wahli & Burkhardt-Holm 2001; Schmidt-Posthaus et al. 2012; Guevara Soto et al. 2016 b). The main causative agents were identified as *Ca*. P. salmonis and *Ca.* C. salmonicola (Schmidt-Posthaus et al. 2012; Guevara Soto et al. 2016 b). Previous studies provided indications that epitheliocystis infections might relate to water temperatures, with seasonal peak occurrences during summer and autumn (Schmidt-Posthaus et al. 2012). However, nothing is known so far about the temporal development of the disease in the course of the year and possible differences between consecutive years.

Therefore, the aims of this study were to investigate whether (i) epitheliocystis is present in brown trout throughout the year, (ii) there are seasonal patterns of prevalence and infection and (iii) differences in prevalence and infection intensities change from year to year. Histology was performed to investigate presence, prevalence and infection intensities of epitheliocystis. Water temperature was measured continously nearby the sampling points during the years 2012-2014.

Material and Methods

Sample collection

The neighbouring Rhone tributaries, the Venoge (length 44 km long) and Boiron (15 km) entering the Lake Geneva, were selected for this study. To investigate the temporal development of the infection during the course of the year, two sampling sites (midriver location Moulin de Lussy and a downriver location Aval CFF) in the river Boiron were sampled in the months of June, July, August, September and November 2013 (Fig. 1, table 1). Fifty to 86 specimens of brown trout were collected by electrofishing from each sampling site every month (total = 500 fish). To investigate inter-year differences, one location in the Venoge (river site Source, headwater location) and one location in the Boiron (Moulin Lussy, midriver location) were selected (Fig. 1, table 2). In 2012, 2013 and 2014, electro fishing was performed to collect brown trout from the different sites in the Venoge and Boiron (Fig. 1). The Venoge was sampled in July and November 2012 and 2013, the Boiron was sampled in July and September 2013 and 2014, with 25 to 86 trout per river site being collected and euthanized by an overdose of tricaine methanesulfonate (MS-222®, Argent Chemical Laboratories, Redmont, USA). At all samplings, lengths of the brown trout were measured. A standard necropsy was performed and macroscopical changes were recorded. On the left body side, the outermost gill branch was removed and fixed in 10% buffered formalin for histopathology.

Water temperature

Water temperature was recorded every 15 min from January 2013 until August 2014 by temperature loggers located in the rivers nearby the fish sampling sites (Fig. 1). In the Venoge the temperature logger was located at the source of the river. In the Boiron, two sampling locations were examined, one midstream and one downstream location. Nearby, three temperature loggers were installed. The first one (Aval Irence) was located upstream the upper sampling point, the second temperature logger (Aval Perceval) was located between the two sampling points and the third logger (Embouchure) was situated near the river mouth into Lake Geneva.

Comment [HS1]: Position of figure 1

Histopathology

Formalin fixed gill samples were trimmed, embedded in paraffin, and sections of 4 µm thickness were cut. Sections were stained with haematoxylin and eosin (H&E). Gill sections were examined by light microscopy for the presence of cysts, and cyst numbers were counted per gill arch (infection intensity). Gill pathology was investigated and the following parameters were encountered: oedema, mainly pronounced as epithelial lifting on the lamellae, infiltration with macrophages and lymphocytes in the area of epitheliocystis cysts and lamellar fusion due to epithelial proliferation, mainly in the basal part of the lamellae. Severity of each parameter was classified as 0 (no lesion), 1 (mild lesions), 2 (moderate lesions) or 3 (severe lesions).

The cyst morphology was used to characterize different types of bacteria (see also Guevara Soto, Vidondo, Vaughan, Seth-Smith, Nufer, Segner, Rubin & Schmidt-Posthaus 2016 a; Guevara Soto et al. 2016 b), *Ca*. P. salmonis (type 1) and *Ca*. C. salmonicola (type 2) or mixed infection (type 3) in all investigated animals. This classification based on morphology was used for all statistical investigations.

Statistics

We first calculated the point prevalence from each river as the percentage of infected fish per total number of investigated animals at the time point of sampling. Differences in prevalence for different months and at different temperatures were assessed by means of logistic regression models.
Infection intensity was measured as number of eptheliocystis cysts per gill arch. The total number per slide was counted. The numbers of cysts per gill arch were not normally distributed. So, differences in the number of cysts per gill arch (infection intensity) between catchments months and the type of pathological lesions (lamellar fusion, oedema, and inflammation), the type of cyst morphology and temperature were explored by means of non-parametric Kruskal-Wallis rank sum tests. All analyses were carried out in R, version 3.1 (https://www.r-project.org/) and packages lme4, rcmdr, car, Rcmdmisc. Data points have been plotted using the jitter option in the R package ggplot2. It's a useful way of handling overplotting caused by discreteness in smaller datasets.

Results

Water temperature

In the Venoge, the fish sampling site and the temperature logger were located close to the source of the river. In this location, water temperatures remained relatively low throughout the observational period, with the daily average ranging from 5.5 °C end of February to 12.7°C beginning of September (Fig. 1).
In the Boiron, water temperature was monitored by three temperature loggers installed in the near vicinities of the sampling sites. Above the midstream sampling site, temperatures ranged from 2 °C end of February to 16.9 °C end of July. At the second logger location, between the two sampling points, water temperature ranged from 2.2 °C mid of January to 18.4°C end of July. At the most downstream location, temperature was in general higher compared to the other locations, with the lowest temperature measured end of February (4.5 °C) and warmest temperature measured mid August (17.8 °C) (Fig. 1). In July, when the first epitheliocystis infections were found in the investigated brown trout, temperatures were exceeding 15-16 °C at all three temperature loggers.

Histopathology of epitheliocystis cysts on the gills

Two different morphologies were distinguished in the epitheliocystis lesions from brown trout in this study based on histology (Fig. 2a and 2b). The first type shows *Ca.* P. salmonis-like cysts with a dark basophilic amorphous center and a clear halo (Schmid-Posthaus et al. 2012, Guevara Soto et al. 2016 a). Infection was associated with a mild to moderate hyperplasia of epithelial cells, subepithelial oedema and mild to moderate infiltration with mainly lymphocytes. The other cyst type shows a lightly basophilic granulated center, associated with a mild edema and mild epithelial hyperplasia, typical for *Ca.* C. salmonicola. Each cyst was classified according to the above mentioned criteria in accordance to Schmidt-Posthaus et al. 2012 and Guevara Soto et al. 2016 a, b and the morphologies of cysts per gill arch were classified as type 1 (*Ca*. P. salmonis), type 2 (*Ca.* C. salmonicola) and mixed (both types 1 and 2) morphology, in the following description as type 3 (Fig. 2, insert).

Comment [HS2]: Position figure 2

Yearly Fluctuations

Prevalence and infection intensities

At both locations in the Boiron, all animals revealed to be negative for epitheliocystis in June. Prevalence then rose to 14-15%, a level that was maintained from August through to the end of the sampling period in November. We only found a very weak evidence of a significant difference in the proportion of infected fish for the month of September with respect to July ($p = 0.0971$). Temperature was not associated with presence of infection ($p=0.200$) and varied from 11.5 to 17.2 ^{o}C.

In contrast to the midriver site, the prevalence at the downriver site, peaked in September, reaching a level of 28%, and afterwards dropping to 12% (Fig. 3).

Comment [HS3]: Position figure 3

Infection intensities, measured as number of cysts per gill arch, did not differ between the sampling sites (Kruskal Wallis rank-sum test, $p=0.433$). When comparing the seasonal occurrence, infection intensity was highest in September (Fig. 4), decreasing again in November, however, this tendency was not significant ($p=0.1353$). Most of the infected fish (22 out of 25) had fewer than 10 cysts per gill arch (Figure 4, Table 1). Three fish collected later in the year (September and November) had up to 35 cysts per gill arch. There was no association between infection intensities and water temperature (Kruskal Wallis rank-sum test, p=0.333).

Comment [HS4]: Position figure 4

The number of cysts per gill arch was highest on average in mixed infections, followed by those for *Ca*. C. salmonicola and it was lowest for the *Ca*. P. salmonis morphology (Kruskal-Wallis rank-sum test, p = 0.02807). *Ca*. C. salmonicola was encountered most often throughout the investigated period (18/22), either in single infections (type 2) or mixed together with Ca. P. salmonis (type 3). In contrast, *Ca*. P. salmonis were only seen in August and September at the midriver site and in September and November at the downstream location, with the lowest numbers in total (4 single infections (type 1) and 8 mixed infections (type 3)). Overall *Ca*. C. salmonicola was more likely to be found in August and September with respect to November (OR±95%CI = 7.28± 1.16-67.82, p = 0.0469).

Comment [HS5]: Position figure 5 and table 1

Inter-year comparison

In each river, the Venoge and Boiron, there were no significant between-year differences in prevalence or infection intensity detectable (logistic regression, p=0.0531 and Kruskal Wallis rank-sum test, p=0.8982). In both rivers, prevalence and infection intensity were increasing from July to November.

Comment [HS6]: Position table 2 and figure 6

Discussion

Epitheliocystis is a disease caused by diverse gram negative intracellular bacteria, found world wide in marine and fresh water fish, but for which we have very little information regarding environmental sources and mechanisms of transmission. In the marine environment, outbreaks can be sudden and mortalities high in young fish transferred from land-based breeding facilities to sea cages for rearing (Crespo, Zarza, Padros & Marin de Mateo 1999; Seth-Smith et al. 2016) or in cultured fish larvae exposed to unfiltered sea water (Katharios, Papadaki, Papandroulakis & Divanach 2008; Katharios et al. 2015). This would imply transmission via the water column, either by freely dispersing bacteria or as passengers in the plankton, possibly as intracellular bacteria of alternative invertebrate hosts. In all cases, the environmental reservoirs are unknown.

Salmonids of the genera *Salmo* and *Salvelinus* are susceptible to epitheliocystis caused by *Ca.* P. salmonis and *Ca*. Brachiomonas cysticola (Draghi et al. 2004, 2010; Mitchell et al. 2010;

Toenshoff et al. 2012; Mitchell et al. 2013; Contador et al. 2015) in marine culture and to *Ca.* C. salmonicola and *Ca.* P. salmonis in fresh water (Mitchell et al. 2010, 2013). Transfer to sea water cages for rearing of *Ca.* C. salmonicola infected Atlantic salmon (*Salmo salar*) leads to loss of infection, indicating a fresh water reservoir for these bacteria. Especially in intensive aquaculture, where less than optimal welfare can lead to increased disease susceptibility (Segner, Sundh, Buchmann, Douxfils, Sundell, Mathieu, Ruane, Jutfelt, Toften & Vaughan 2012), infected con-specifics could serve as a source for recurrent infections, although wild populations cannot be excluded. One possibility investigated was cleaner wrasse utilised in salmon farms to reduce parasite loads in the cultured salmon. In an elegant study, these were indeed found to suffer from epitheliocystis, but the infectious agent was *Ca.* Similichlamydia labri, which has not been found in cultured Atlantic salmon, although related *Ca.* Similichlamydia species cause epitheliocystis in both marine cultured striped trumpeter (Stride et al. 2013a) and brackish and fresh water cultured barramundi (Stride et a. 2013b) in the Southern hemisphere.

To gain insight into these questions, we have taken advantage of the geographical situation of Switzerland, distant from the nearest marine environments, yet source of the headwaters of several major European river systems, including the Rhine, which flows into the Atlantic and the Rhone which drains into the Mediterranean. A wild native brown trout (*Salmo trutta*) population is susceptible to epitheliocystis caused by *Ca.* C. salmonicola and *Ca.* P. salmonis (Schmidt-Posthaus et al. 2001, 2012; Guevara Soto et al. 2016 a, b), which indeed appears to derive from reservoirs, either within the wild brown trout population and/or from alternative hosts. As part of our efforts to narrow our search for an environmental reservoir, we have followed the appearance of epitheliocystis in the wild brown trout population within two neighbouring moderate sized tributaries of the Rhone, the Venoge and Boiron, both throughout the summer and autumn at several sites and over three years at selected sites. Focus was on the most prominent chlamydial species identified in Swiss brown trout, *Ca.* P. salmonis and *Ca.* C. salmonicola (Guevara Soto et al. 2016 a, b). These two species were previously shown to be common in the Venoge and Boiron and can be distinguished morphologically by typical microscopical features (Schmidt-Posthaus et al. 2012, Guevara et al. 2016 a). In both these studies the histological appearance could be confirmed by electron microscopy. Therefore, in this study, discrimination was done by histology only.

In the past, it was hypothesized that epitheliocystis infections correlate to water temperature with a seasonal occurrence shortly after the summer temperature peak (Schmidt-Posthaus et al. 2012). Although infections are not significantly correlated to water temperature, there appears to be a seasonal occurrence with peak values shortly after the summer months. As temperature has an influence on the fish immune system (Köllner and Kotterba 2002; Bowden 2008; Cheng, Chenga, Chena & Chen 2009; Jokinen, Salo, Markkula, Rikalainen, Arts & Browman 2011), temperature dependence of disease in association with suboptimal immune functions could be suspected also in epitheliocystis infections. No epitheliocystis could be detected at the beginning of summer. This indicates that brown trout accumulate infections during the course of the year and that bacteria appear not to be present in the wild brown trout population during winter and spring. Indicative for this suggestion is also the already decreasing prevalence in November in the Boiron, Aval CFF. However, the nature of the reservoir during this time of the year is still unknown. As chlamydial species inducing epitheliocystis are still not cultivable, experimental infections to test for possible transmission routes are not possible. Therefore, we still do not know if these bacteria are directly transferable between brown trout. Possibly, a critical temperature has to be surpassed before a chlamydial infection can be established.

This hypothesis would be supported by the fact that in the Venoge brown trout sampled in July were still negative, both in 2012 and 2013. In the Venoge, the sampling location was situated far upstream with water temperature remaining lower compared to the locations sampled in the Boiron. Once the epitheliocystis infection is established in the brown trout, a temperature drop does not seem to induce the clearance of infection, at least up until the end of autumn. Given that the levels of infection prevalence and intensities appear to be maintained from year to year, the reservoir must be well established and stable. This is an important finding, as it provides the basis for planning a more extensive environmental investigation of these rivers, throughout the year and including both other fish and also possible invertebrate hosts.

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Figure legends

Figure 1. . Map of location of Boiron and Venoge. Both rivers drain into Lake Geneva. Sampling sites are marked as black points (Vs1 = Venoge, Source; Bs1 = Boiron, Moulin Lussy; Bs2 = Boiron, Aval CFF), location of temperature loggers are marked as dark green rectangles (Vt1 = Venoge, Source; Bt1 = Boiron Aval Irence; Bt2 = Aval Perceval; Bt3 = Boiron, Embouchure). Temperature curves at the three locations in the Boiron and the one location in the Venoge, the blue line indicates the mean daily water temperature

Figure 2. a. Brown trout (*Salmo trutta*), gills, *Ca.* P. salmonis cysts characterized by condensed basophilic intracellular material surrounded by a clear halo; up to 20 µm in diameter. Open arrowheads indicate chlamydial cysts. In the surrounding tissue there is oedema in the subepithelial area and infiltration with lymphocytes and macrophages (arrow), star indicates epithelial hyperplasia. HE, bar = 50 µm. b. *Ca.* C. salmonicola cysts characterized by granular loosely arranged material filling the whole cyst space, up to 20 µm in diameter. HE, bar = 50 µm. Insert: mixed infection with both, *Ca.* P. salmonis (open arrowhead) and *Ca.* C. salmonicola (closed arrowheads).

Figure 3. Prevalence (percentage of infected animals per time point of sampling and location), blue line indicates midriver location at the Boiron (Moulin de Lussy), red line indicates downriver location (Aval CFF).

Figure 4. Box plots of number of cysts per gill arch (infection intensity) per month in the Boiron, both sampling sites are combined. There were no infected fish in the month of June. Data points are shown using the jitter option in the R package ggplot2.

Figure 5. Box plots of number of cysts per gill arch (infection intensity) in dependence of the cyst type showing highest infection intensity in mixed infections. Cyst type 1 = morphology typical for *Ca*. P. salmonis, type 2 = morphology typical for *Ca.* C. salmonicola, type 3 = mixed infection. Data points are shown using the jitter option in the R package ggplot2.

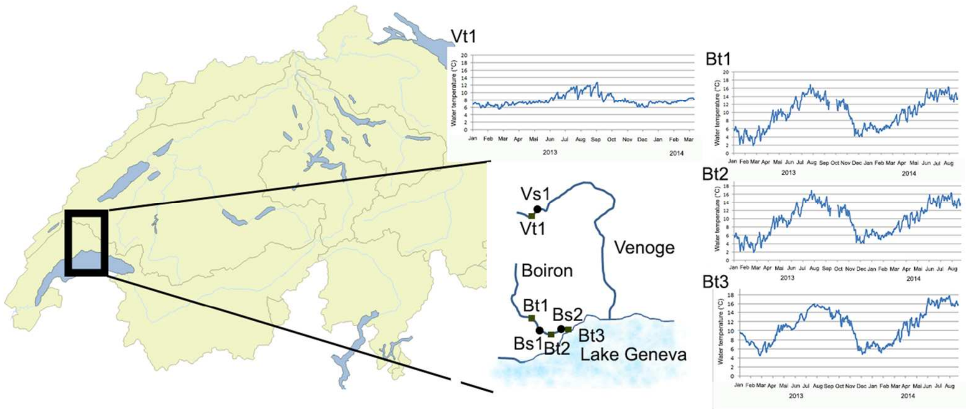
Figure 6. Inter-year comparison of infection intensities (number of cysts per gill arch) between 2012 and 2013 in the Venoge and between 2013 and 2014 in the Boiron. Data points are shown using the jitter option in the R package ggplot2.

Table 1: Prevalence and infection intensity of epitheliocystis in brown trout originating from the Boiron sampling sites in 2013. Cyst type 1 = Ca. P. salmonis, cyst type 2 = Ca. C. salmonicola, cyst type 3 = mixed.

River	River site	Location	Year	Date of sampling	n	Prevalence (%) Histology (n)	Intensity (n cysts) median	Cyst type
Boiron	Moulin de Lussy	Midriver site	2013	05.06.	86	0 (0)	0	0
				08.07.	50	4 (2)	2	2
				22.08.	50	16 (8)	2	1,2,3
				12.09.	50	12 (6)	2	1,2
				08.11.	62	16 (10)	3	2
	Aval CFF	Downriver site	2013	05.06.	50	0 (0)	0	0
				22.08.	50	8 (4)	1.5	2
				12.09.	50	28 (14)	7	3
				08.11.	52	12 (6)	2	1,2,3

Table 2: Prevalence and infection intensity of epitheliocystis in brown trout originating from the Venoge sites, sampling 2012 and 2013 and Boiron sites, sampling 2013 and 2014

River	River site	Location	Year	Date of sampling	N	Prevalence (%) Histology (n)	Intensity (n cysts) median
Venoge	Source	Headwater site	2012	12.07.	26	0 (0)	0
				15.11.	26	26 (7)	2
			2013	12.07.	25	0 (0)	0
				29.11.	26	58 (15)	3
Boiron	Moulin Lussy	Midriver site	2013	08.07.	50	4 (2)	2
				12.09.	50	12 (6)	2
			2014	07.07.	25	8 (2)	2.5
				08.09.	25	12 (3)	4



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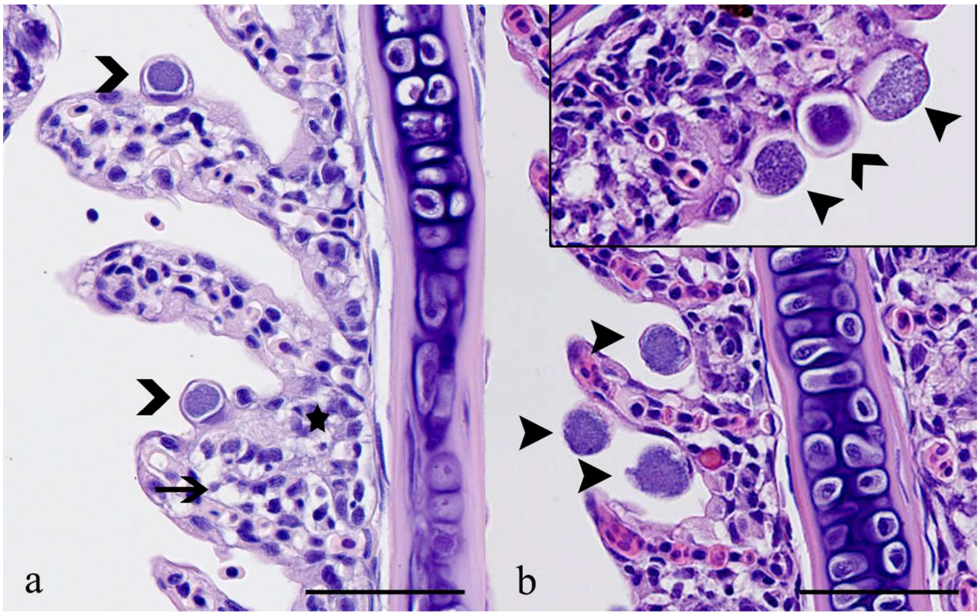

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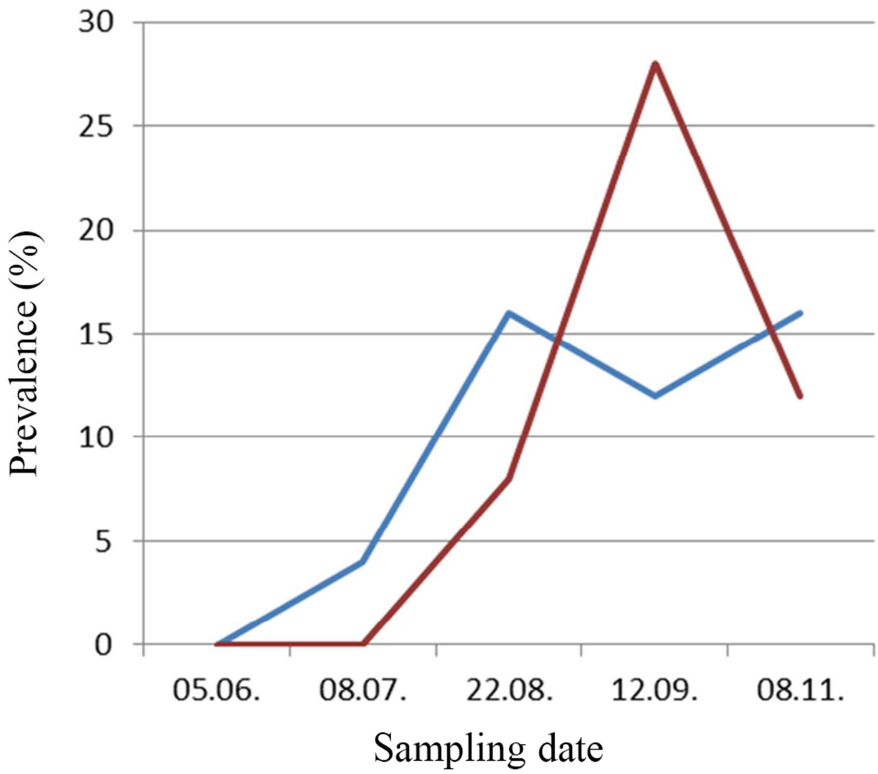

Figure 3. Prevalence (percentage of infected animals per time point of sampling and location), blue line indicates midriver location at the Boiron (Moulin de Lussy), red line indicates downriver location (Aval CFF).

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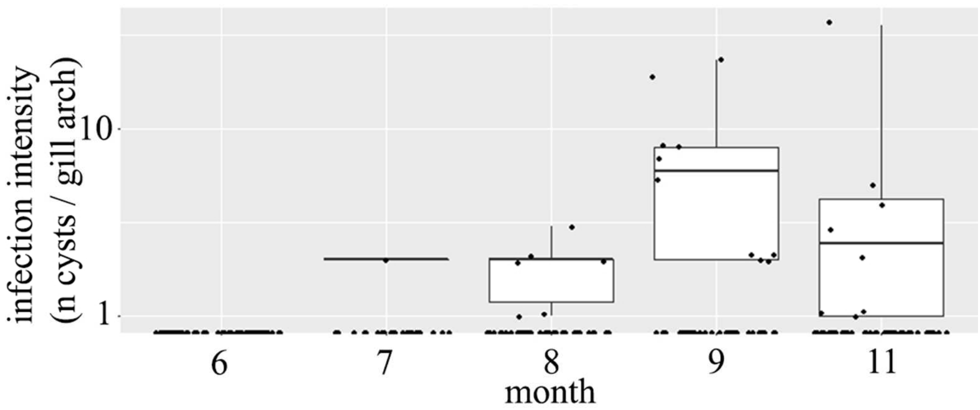

Figure 4. Box plots of number of cysts per gill arch (infection intensity) per month in the Boiron, both sampling sites are combined. There were no infected fish in the month of June. Data points are shown using the jitter option in the R package ggplot2.

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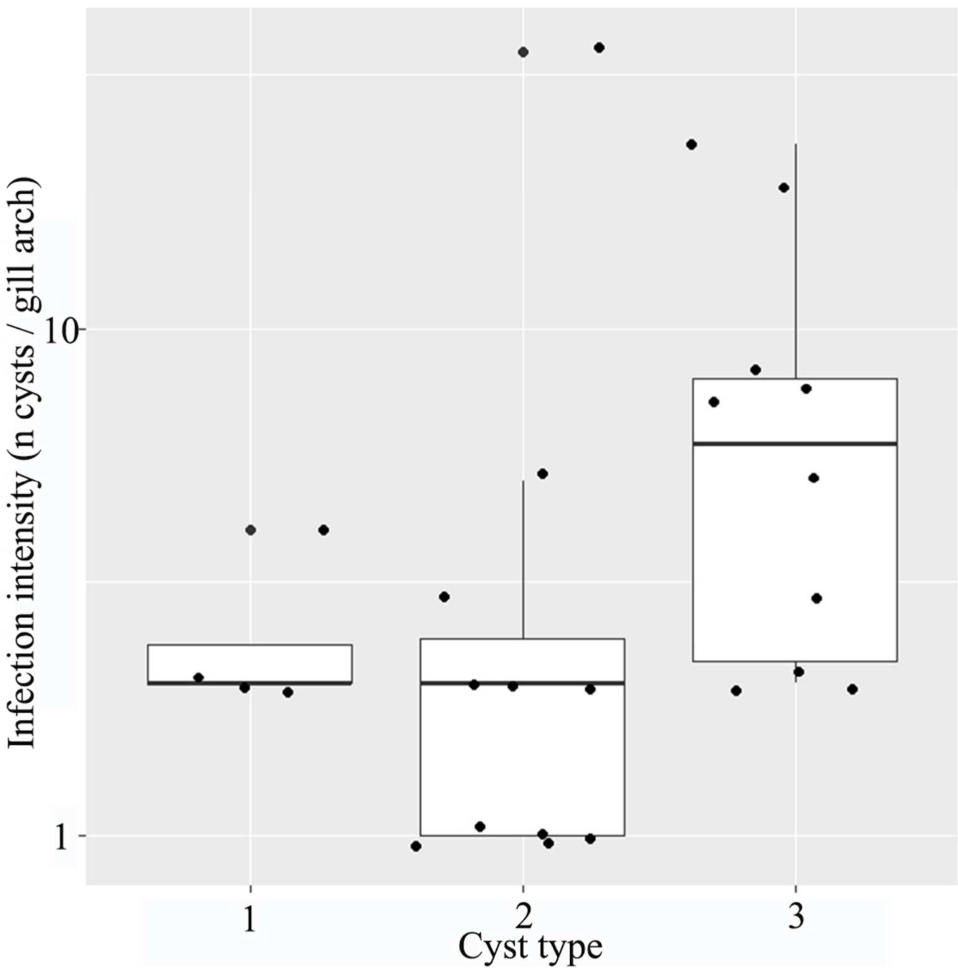

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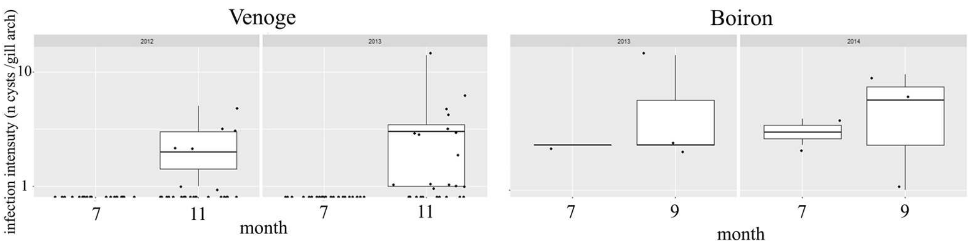

Figure 6. Inter-year comparison of infection intensities (number of cysts per gill arch) between 2012 and 2013 in the Venoge and between 2013 and 2014 in the Boiron. Data points are shown using the jitter option in the R package ggplot2.

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